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Research paper

Infection of chicken bone marrow mononuclear cells with subgroup J avian leukosis virus inhibits dendritic cell differentiation and alters cytokine expression

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ABSTRACT

Subgroup J avian leukosis virus (ALV-J) is an oncogenic retrovirus known to induce tumor formation and immunosuppression in infected chickens. One of the organs susceptible to ALV-J is the bone marrow, from which specialized antigen-presenting cells named dendritic cells (BM-DCs) are derived. Notably, these cells possess the unique ability to induce primary immune responses. In the present study, a method of cultivating and purifying DCs from chicken bone marrow *in vitro* was established to investigate the effects of ALV-J infection on BM-DC differentiation or generation. The results indicated that ALV-J not only infects the chicken bone marrow mononuclear cells but also appears to inhibit the differentiation and maturation of BM-DCs and to trigger apoptosis. Moreover, substantial reductions in the mRNA expression of TLR1, TLR2, TLR3, MHCI, and MHCII and in cytokine production were detected in the surviving BM-DCs following ALV-J infection. These findings indicate that ALV-J infection disrupts the process of bone marrow mononuclear cell differentiation into BM-DCs likely *via* altered antigen presentation, resulting in a downstream immune response in affected chickens.
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1. Introduction

Avian leukosis virus (ALV) belongs to the family Retroviridae, subfamily Orthoretrovirinae, and genus *Alpharetrovirus*. In chickens, ALVs can be further classified into six subgroups: A, B, C, D, E, and J. Notably, ALV-J, which was first isolated in chickens in 1988, has been shown to induce myeloid leukosis in broiler chickens (Payne et al., 1992; Payne and Nair, 2012). In fact, the high mortality rate associated with tumour formation and progression as well as with decreased fertility in these animals has caused major economic losses in the poultry industry worldwide, including that in China (Gao et al., 2012). Although detection of ALV-J infection in chickens was first reported in 1997, ALV-J has become more widespread in both commercially and locally bred chickens in various regions of the country in recent decades (Cui et al., 2006).

Furthermore, several ALVs, including subgroup J, have been reported to function as important co-infection factors for various avian diseases, including Marek's disease, reticuloendotheliosis, and others (Abolnik and Wandrag, 2014; Cui et al., 2009; Isfort et al., 1994). Concomitant infection with an exogenous ALV likely enhances the susceptibility of chickens to these diseases *via* immunosuppression (Gao et al., 2015); however, the underlying mechanism of immunosuppression by ALV infection remains unclear. Notably, the incubation period of ALV-J also remains unclear, and immunosuppression is often observed to occur before the onset of infection-related symptoms, suggesting that ALV-J may affect the function of the host immune system much earlier than previously thought. Thus, further investigation of effects of ALV-J infection on the host immune system will be helpful in preventing and controlling these diseases.

Dendritic cells (DCs) were first described as Langerhans cells in the skin in 1868. In 1973, Steinman and Cohn identified DCs from mouse spleen tissue and named them based on their typical morphology (Banchereau and Steinman, 1998; Steinman and Cohn, 1973). Notably, DCs are antigen-presenting cells of the immune system, and they possess the unique capacity to initiate primary immune responses (Austyn, 1996; Avila-Moreno et al., 2006; Steinman, 1991). As central regulators of innate and adaptive immunity, DCs can not only stimulate T cells but also express several different pathogen recognition receptors,

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such as Toll-like receptors (TLRs), which facilitate antigen presentation (Mellman and Steinman, 2001). DCs also secrete cytokines and chemokines in response to specific pathogens, thus altering the microenvironment in order to promote the maturation of various cell types (Izquierdo-Useros et al., 2014). The role of these cells in the immune response makes them invaluable when studying the effects of any foreign pathogen, including viruses.

Research utilizing chicken-derived DCs was initiated later than that using mammalian DCs, and reproducible methods for generating and characterizing chicken DCs have only been established since 2010 (Wu et al., 2010). Notably, infection of cultured DCs with avian influenza virus (AIV) was reported to cause a strong increase in cytokine expression and enhancement of DCs activation, which may trigger deregulation of immune responses (Vervelde et al., 2013). However, unlike AIV, ALV primarily spreads through vertical transmission, and the associated immune response may involve a different mechanism. As a retrovirus, it is also possible that once the ALV provirus is integrated into the host genome following hen to egg transmission, it may alter gene expression and affect embryonic development and differentiation in a variety of cell types (Pajer et al., 2006). Therefore, it is a hypothesis that ALV-J-mediated deregulation of specific genes involved in the differentiation and/or development of immune cells, especially DCs, could result in irregular maturation and an abnormal immune response.

In the present study, we investigated the effects of ALV-J infection on chicken bone marrow-derived DC (BM-DC) development and found that ALV-J infection does in fact inhibit normal BM-DC maturation and function. Our data indicate that this virus not only alters the maturation and apoptosis rates of BM-DCs but also affects the mRNA expression of TLRs and various cytokines. We believe these changes would significantly influence chicken BM-DC function *in vivo* and could result in immunosuppression. Furthermore, we established and optimized an *in vitro* method for cultivating and purifying DCs from chicken bone marrow, which may be beneficial in future studies to explore the interactions between chicken BM-DCs and other avian viruses.

2. Materials and methods

2.1. Animals

One-day-old specific-pathogen-free (SPF) chickens were hatched from SPF eggs (Merial Vital Laboratory Animal Technology Company, Beijing, China). All of the animal research procedures utilized in this study were approved by and conducted under the guidance of the South China Agricultural University (SCAU) Institutional Animal Welfare Ethics and Use Committee.

2.2. Virus strain

Subgroup J ALV strain NX0101 was kindly provided by Professor Cui Zhizhong (College of Animal Science and Technology, Shandong Agriculture University, Taian, Shandong, China). Notably, NX0101 is a myeloma-inducing type of ALV-J (Cui et al., 2006).

2.3. Isolation of and culture of chicken BM-DCs

BM-DCs were isolated using previously published methods with some modifications. Briefly, chickens were euthanized at 1 week of age. Then, both ends of their femurs were cut, and the marrow was flushed with PBS. Mononuclear cells were isolated from the marrow using chicken lymphocyte separation medium (Solarbio, Beijing, China) and then cultured in cell culture plates (1×10^6 cells/mL) with RPMI-1640 complete medium (Gibco, CA, USA) containing 10% heat-inactivated chicken serum (Gibco) and optimal concentrations (30–50 ng/mL) of rhGM-CSF and rhIL-4 (PeproTech, NJ, USA). The medium was refreshed every 3 days, on average. Cells were cultured at 39 °C with 5% CO_2 for 10 days. On the 10th day of culture, cells were stimulated with 200 ng/mL lipopolysaccharide (LPS) (Sigma, Santa Clara, CA, USA) (Wu et al., 2010).

BM-DCs were identified using an inverted microscope and scanning electron microscopy (Fei-XL30 ESEM, Fei, Hillsboro, USA). Flow cytometry (FC500MCL/MPL, Beckman Coulter, USA) was used to evaluate the cell surface expression of the widely accepted DC maturation markers CD11c and CD86, which are markedly increased on the surface of LPS-stimulated DCs (Wu et al., 2010). The LPS-stimulated BM-DCs were then collected and analysed using PE-conjugated anti-mouse CD11c and FITC-conjugated anti-mouse CD86 monoclonal antibodies (Affymetrix eBioscience, CA, USA).

2.4. Infection of BM-DCs with ALV-J

After BM-DCs were cultured for 2 days, they were infected with a dose of 10^4 TCID50/mL of ALV-J NX0101 strain in serum-free medium, and the cells were incubated for an additional 2 h at 39 °C, 5% CO_2. These ALV-J-infected cells, together with mock-infected cells that were incubated in serum-free medium, were washed with PBS, and then fresh medium was added. After the cells were cultured at 39 °C, 5% CO_2 for 7 days, they were stimulated with LPS for 24 h.

2.5. ALV-J infection analysis

The percentage of mature BM-DCs among the infected cells was determined *via* flow cytometry for CD86 and CD11c expression as outlined above. The supernatants were harvested from cultures of ALV-J-infected and mock-infected control BM-DCs at various stages during the first 1–7 days post-infection (d.p.i.), and the presence of ALV-J was detected using an ALV p27 enzyme-linked immunosorbent assay (ELISA; ALV antigen test kit, IDEXX, ME, USA). The positive sample results were calculated according to the manufacturer's instructions.

To further verify viral infection of the BM-DCs, DNA was isolated from the cells (1–7 d.p.i.) using a commercial kit according to the manufacturer's instructions (Omega Bio-Tek, Norcross, GA, USA), and the proviral ALV-J DNA was detected by PCR. All samples were stored at −80 °C. PCR was performed using Taq DNA polymerase (New England Biolabs, Ipswitch, USA) with the following conditions: 95 °C for 5 min, followed by 35 cycles of 95 °C for 30 s, 55 °C for 30 s, and 72 °C for 1 min. A final extension was performed at 72 °C for 10 min. The specific ALV-J PCR primers used in this study (Table 1) were designed based on the published target sequence (GenBank ID: Z46390.1), and the purified products were sequenced to verify the identity of the amplification fragments.

2.6. Measuring apoptosis in BM-DCs

The level of apoptosis was measured in the mock-infected and ALV-J-infected BM-DCs at 7 d.p.i. using the DeadEnd Fluorometric terminal deoxynucleotidyl transferase dUTP nick-end labelling (TUNEL) System (Promega, WI, USA). Briefly, ALV-J-infected and mock-infected cells were collected after LPS stimulation (3 h, 6 h, 12 h, and 24 h), and then the collected cells were diluted and co-stained with DAPI and TUNEL. The subsequent microscopic examination was performed according to the manufacturer's instructions using a fluorescence microscope (Leica DMI 4000B, Leica, Germany). The ratio of apoptotic cells was determined by quantifying the number of fluorescent cells per field (Mukherjee et al., 2012). Three fields were counted for each sample, and there were 3 parallel samples in each experimental group.

2.7. Analysis of TLR and cytokine expression

We isolated mRNA from ALV-J- and mock-infected BM-DCs at 7 d.p.i. after 24 h of LPS stimulation using a commercial kit according to the manufacturer's instructions (Omega Bio-Tek). Quantitative real-time PCR (qRT-PCR) was performed using SYBR Green PCR Master Mix

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